Bcl-2 and Bax Exert Opposing Effects on Ca\(^{2+}\) Signaling, Which Do Not Depend on Their Putative Pore-forming Region*

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vitro by Bcl-2 and Bax is unknown but could be due, at least in part, to differences between the polar residues of the α5-α6 regions (reviewed in Ref. 16). In this work, we have employed aequorin-based recombinant probes targeted to specific intracellular localizations and used two cellular models (stable inducible clones versus transient expression) to evaluate the effect of Bax overexpression on subcellular Ca2+ homeostasis. We then analyzed the role of the presumed channel-forming α5-α6 helices of Bcl-2 and Bax in Ca2+ mobilization by using two chemical constructs in which the putative pore domains of the proteins were mutually swapped.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Ionomycin, histamine, ATP, digoxin, N-acetyl-L-sphingosine (C2-ceramide), Geneticin, hygromycin B, and doxycycline were purchased from Sigma-Aldrich, and coelenterazine was purchased from Cellbio, Euroclone) in 75-cm2 Falcon flasks. All cells were maintained reconstituting erAEQmut with high efficiency the luminal \[Ca^{2+}\]o ft h e

**Generation of the Bax Tet-off Cell Line—**Bax Tet-off cell lines were generated by co-transfecting pTRE-Bax with pTK-Hyg at a ratio of 20:1, using the calcium phosphate co-precipitation method, into a HeLa Tet-off cell line (HeLa cell line stably transfected with pTet-off regulatory construct) (18). The wild-type human Bcl-2 and Bax cDNAs and the chimeric mtAEQ-mut) or the cytosolic aequorin probe (cytAEQ) (18). For subcellular localizations and used two cellular models (stable inducible aequorin-based recombinant probes targeted to specific intracellular compartments). The most striking result was the drastic reduction of the mitochondrial Ca2+ signals occurring in the cytoplasm and in organelles acting as the source (ER) or target (mitochondria) of Ca2+. We investigated the response to ATP, which acts on Gαi-coupled plasma membrane receptors and causes the production of IP3, thus releasing Ca2+ from the ER, followed by sustained influx from the extracellular medium through plasma membrane Ca2+ channels. In these experiments, as shown in Fig. 1, HeLa cells were either co-transfected with Bax and the aequorin chimera of interest (fraces labeled Bax) or transfected only with the aequorin probe (controls). Where indicated, the cells were challenged with ATP.

**Immunofluorescence Analysis—**Transfected HeLa cells were fixed in a cold (−20%) methanol:acetone (1:1) solution for 10 min at room temperature and permeabilized with 0.2% Triton X-100 for 10 min. After three washes with PBS (5 min each), immunodetection was carried out using Texas Red-conjugated goat anti-rabbit IgG (diluted 1:200 in 3% BSA) for 1 h. After three washes with PBS (5 min each), immunodetection was carried out using Texas Red-conjugated goat anti-rabbit IgG (diluted 1:200 in 3% BSA).

**Microscopic Analyses—**Mitochondrial morphological analyses were performed 36 h post-transfection. 24-mm coverslips containing the cells were placed in a thermostatted Leiden chamber (model TC-202A, Medical system Corp.) on the stage of a Zeiss Axiosvert 200 inverted microscope (Carl Zeiss, SPA, Milano, Italy) equipped with epifluorescence and piezoelectric motorization of the objective (Physik Instrumente). Fluorescent images were captured by a cooled charge-coupled device camera (CoolSnap, Roper Scientific, Inc.) and analyzed using MetaMorph 5.0 software (Universal Imaging Corp.). Z-series images were deconvolved using Exhaustive Photonic Reassign (EPR) software, a point spread function-based deconvolution algorithm developed by the University of Massachusetts Imaging Group (20, 21), running on a Linux-based PC.

**Apoptosis Analyses—**Apoptosis was evaluated by counting apoptotic nuclei (fragmented nuclei) among GFP-positive cells using 7-aminomethylemercuric-bis-chromotropic acid (Sigma-Aldrich) as a cytotoxicity assay (19). After two washes with PBS, the cells were permeabilized with Triton 0.2% in PBS for 10 min and washed again with PBS. Samples were then incubated with 7-aminactinomycin D at room temperature for 15 min. The percentage of apoptotic cells was calculated from at least 400 GFP-negative cells from three or more independent experiments. For C2-ceramide (作息 Bcl-2 and Bax transient expression) to evaluate the effect of Bax overexpression on subcellular Ca2+ homeostasis in transiently transfected HeLa cells, i.e., the same cell model in which the effect of Bcl-2 on Ca2+ signaling was detected and analyzed previously (4). We used targeted chimeras of the Ca2+-sensitive photoprotein aequorin to monitor the dynamics of agonist-dependent (Ca2+) changes occurring in the cytoplasm and in organelles acting as the source (ER) or target (mitochondria) of Ca2+ signals.

**RESULTS**

**Transient Overexpression of Bax in HeLa Cells Drastically Reduces Mitochondrial Ca2+ Responses but Leaves ER Ca2+ Levels Unaffected—**The first task of this work was to evaluate the effect of Bax overexpression on subcellular Ca2+ homeostasis in transiently transfected HeLa cells, i.e., the same cell model in which the effect of Bcl-2 on Ca2+ signaling was detected and analyzed previously (4). We used targeted chimeras of the Ca2+-sensitive photoprotein aequorin to monitor the dynamics of agonist-dependent (Ca2+) changes occurring in the cytoplasm and in organelles acting as the source (ER) or target (mitochondria) of Ca2+ signals.

We investigated the response to ATP, which acts on Gαi-coupled plasma membrane receptors and causes the production of IP3, thus releasing Ca2+ from the ER, followed by sustained influx from the extracellular medium through plasma membrane Ca2+ channels. In these experiments, as shown in Fig. 1, HeLa cells were either co-transfected with Bax and the aequorin chimera of interest (fraces labeled Bax) or transfected only with the aequorin probe (controls). Where indicated, the cells were challenged with ATP.

The most striking result was the drastic reduction of the mitochondrial Ca2+ rise evoked by agonist stimulation in Bax overexpressing cells; peak value 59 ± 9 μM in Bax cells versus 87 ± 9 μM in control cells (n = 7, n = 11, respectively, p < 0.01 (Fig. 1B)), i.e. a 32% decrease in amplitude versus control cells. In contrast, a small (9%), albeit statistically significant reduction was detected in the cytosolic Ca2+ peak (peak value 2.1 ± 0.05 μM, n = 24 in Bax cells versus 2.3 ± 0.06 μM, n = 38 in control cells, p = 0.01 (Fig. 1A)). We then focused our attention on the ER store, given that mitochondria depend on ER Ca2+ release for the rapid accumulation of Ca2+ in the matrix during
These results showed that Bax per se reduces mitochondrial and, to a lesser extent, cytosolic Ca\(^{2+}\) signals. Our findings were surprising given that a reduction of ER Ca\(^{2+}\) levels and the ensuing reduction of cellular Ca\(^{2+}\) signals represent key findings in Bel-2-overexpressing cells. In agreement with this view (and in apparent contrast with our results), a reduction in ER Ca\(^{2+}\) levels and cellular Ca\(^{2+}\) signals was detected also in cells in which the Bax/Bak genes were ablated (11). Thus, we considered the possibility that reduction of Ca\(^{2+}\) signals is not a direct primary effect of Bax but, rather, that after prolonged high-level expression of Bax, cells have significantly progressed into apoptosis and the down-regulation of Ca\(^{2+}\) signaling is part of the final stages of the apoptotic phenotype. In support of this possibility, we observed a high percentage of apoptotic cells in our experimental conditions, i.e. cells transiently expressing Bax under the control of a strong cytomegalovirus-derived promoter (Fig. 1D). Moreover, we observed that the reduction of mitochondrial agonist-evoked Ca\(^{2+}\) signals was proportional to Bax expression. Taking the [Ca\(^{2+}\)]\(_{er}\) rise of control cells as 100%, the agonist-evoked [Ca\(^{2+}\)]\(_{er}\) peak was reduced in Bax-transfected cells to 82% and 68% at 24 and 36 h post-transfection, respectively (Fig. 1E). In this context, to reveal the primary effect of Bax on Ca\(^{2+}\) signaling, we proceeded to the generation of a new experimental model in which Bax expression can be finely tuned and its early pro-apoptotic effects can thus be assessed.

Characterization of the Inducible Bax Clone—We generated a cell clone expressing Bax in an inducible manner by stably transfecting a HeLa clonal cell line (HeLaTet-off) expressing the tetracycline regulatory element with an expression vector in which the Bax cDNA was placed downstream of a tetracycline-repressible promoter. Stable clones were generated by hygromycin selection and screened by Western blotting for conditioned Bax expression. One clone was selected for the following studies that shows a finely tunable up-regulation of Bax expression after induction by doxycycline withdrawal (up to a 2-fold increase after 48 h; see Fig. 2A). Bax induction did not alter the protein level of endogenous anti-apoptotic Bcl-2 protein, and thus the rheostat of anti-apoptotic and pro-apoptotic proteins in these cells was modestly affected and depended mainly on the protein level of pro-apoptotic Bax (Fig. 2A).

Immunostaining analyses revealed the enhancement of the Bax signal, as well as its re-localization to mitochondria upon doxycycline withdrawal (Fig. 2A). As a consequence of Bax expression, a modest enhancement of apoptotic cell death was detected 48 h after induction (10% in −Dox cells versus 2% in +Dox cells (Fig. 2B)), which correlated with a reduction of mitochondrial potential measured by tetramethylrhodamine methyl ester staining intensity (13% reduction in −Dox cells as compared with +Dox cells (Fig. 2C)).

Bax Induction Is Associated with a Transient ER Ca\(^{2+}\) Overload and an Increase in Mitochondrial Ca\(^{2+}\) Signals—We then evaluated ER Ca\(^{2+}\) homeostasis in the inducible Bax clone. Twenty-four hours after induction of Bax expression, reconstitution of erAEQmut with coelenterazine was performed in Tet-off cells (as well as in cells maintained under tetracycline gene repression, i.e. the controls of this experiment). After transferring the cells to the lumimoteur chamber, perfusion was initiated with a KRB/EGTA buffer, which was then switched to KRB/EGTA. In the latter condition, [Ca\(^{2+}\)]\(_{ER}\) gradually increased, reaching in ~2 min a steady-state plateau value that was markedly different in Bax-induced and control cells. As shown in Fig. 3A, Bax induction was associated with an increase of steady-state [Ca\(^{2+}\)]\(_{ER}\) values (335 ± 13 μM, n = 17 in −Dox cells versus 255 ± 8 μM, n = 22 in +Dox cells, p ≤ 0.0001) (Fig. 3A). Kinetic analysis of IP\(_3\)-induced Ca\(^{2+}\) release from the
**Fig. 2.** Characterization of the Bax-inducible stable clone. A, analysis of Bax expression in stable Bax-expressing clone generated from HeLa Tet-off cells. Western blot analyses were performed in the presence of doxycycline (+) and 24 or 48 h after doxycycline withdrawal (−24 h and −48 h, respectively). The same blot was successively hybridized with Bax, Bel-2, and β-tubulin antibodies. Immunolocalization of Bax in Bax/HeLaTet-off cells before (+Dox) and after (−Dox) Bax induction (48 h). B, counting of apoptotic bodies (shown as percentage) was performed using DNA dye 7-amino actinomycin D staining in Bax/HeLaTet-off cells before and after Bax induction (48 h). Cells with nuclear blebbing considered as apoptotic cells are shown in the inset. C, mitochondrial tetramethylrhodamine methyl ester (TMRM) loading intensity (reflecting mitochondrial potential) in Bax/HeLaTet-off cells before and after Bax induction (48 h). n, number of cells analyzed.

**Fig. 3.** Subcellular Ca2+ homeostasis in the Bax-inducible clone. [Ca2+] measurements were performed before (+Dox) and 24 h after (−Dox) induction. A, as in Fig. 1C, cells transfected with erAEQ were first perfused with KRB/100 μM EGTA. Where indicated, EGTA was replaced with 1 mM CaCl2 that was maintained until steady-state [Ca2+]ER was reached. B, when the steady-state [Ca2+]ER was reached, HeLa cells were stimulated with 100 μM histamine added to KRB/1 mM Ca2+. C, [Ca2+]ER in Bax cells (−Dox), 24 and 48 h post-induction, is represented as a percentage of the steady state of control cells (+Dox). D, mitochondrial Ca2+ homeostasis. Cells transfected with mtAEQ-wt were perfused with KRB/Ca2+ and stimulated with 100 μM histamine where indicated; +Dox and −Dox are represented as black and gray traces, respectively. All other conditions are as described for Fig. 1.
ER revealed a greater initial Ca\textsuperscript{2+} release upon histamine stimulation in Bax-induced cells (Fig. 3B). Interestingly, the [Ca\textsuperscript{2+}]\textsubscript{ER} increase upon Bax induction appeared to be transient; 48 h after the beginning of Bax induction, the [Ca\textsuperscript{2+}]\textsubscript{ER} value became similar to control cells (240 ± 12 μM, n = 7 in −Dox cells) (Fig. 3C), thus mimicking the situation of the high-level transient expression of Bax (Fig. 1). We then investigated mitochondrial Ca\textsuperscript{2+} responses. In agreement with the ER data, a larger [Ca\textsuperscript{2+}]\textsubscript{m} rise was observed in Bax-induced cells upon histamine stimulation (peak amplitude 4 ± 0.2 μM, n = 15 in −Dox cells versus 2.5 ± 0.15 μM, n = 15 in +Dox cells, p < 0.01) (Fig. 3D). These results indicate that, as suggested by experiments carried out in other cells models (the Bax/Bak knock-out lines) (11), Bax exerts an opposite effect to Bcl-2 on the state of filling of the ER Ca\textsuperscript{2+} store (increased in the former, reduced in the latter case). In turn, a greater filling of the ER store may allow mitochondrial Ca\textsuperscript{2+} overload and ensuing recruitment of these organelles into apoptotic responses, thus representing an important initiating step in the apoptotic process triggered by this protein.

Establishment of the Role of the Putative Pore-forming Domain (α5-6) in Ca\textsuperscript{2+} Mobilization by Bax and Bcl-2—An apparent explanation of our results is that the different effects of Bcl-2 and Bax on Ca\textsuperscript{2+} signaling of these proteins are due to differences in the putative pore-forming domain (α5-6) present in both the pro- and anti-apoptotic family members. To investigate this issue, we used two mutant constructs in which the α5-6 helices of Bcl-2 and Bax were mutually swapped (Fig. 4A) (19) and performed a comparative analysis of subcellular [Ca\textsuperscript{2+}] changes occurring in HeLa cells transiently co-transfected with the appropriate aequorin chimera and either Bcl-2/Bax(α5-6), Bax/Bcl-2(α5-6), wild-type Bcl-2, or wild-type Bax. The numbers indicate amino acid positions. BH, Bcl-2 homology domains; TM, transmembrane domain. B–D, subcellular Ca\textsuperscript{2+} homeostasis analyses using erAEQmut (B), mtAEQ (C), and cytAEQ (D). [Ca\textsuperscript{2+}]\textsubscript{ER} steady state (B), [Ca\textsuperscript{2+}]\textsubscript{m} (C), and [Ca\textsuperscript{2+}]\textsubscript{c} responses (D) evoked by agonist stimulation are represented as percentage of control cells, averaging results from at least five independent experiments ± S.E.
Bcl-2 and Bax Effects on Ca\textsuperscript{2+} Signaling

Bcl-2 and Bax Ca\textsuperscript{2+} measurements were performed 36 h after transfection, and the results are shown in Fig. 4 as the mean of the [Ca\textsuperscript{2+}]\textsubscript{ER} steady-state levels (Fig. 4B) and of the agonist-evoked Ca\textsuperscript{2+} signals in the mitochondria (Fig. 4C) and cytosol (Fig. 4D) expressed as a percentage of those of control cells.

These analyses revealed a similar reduction of [Ca\textsuperscript{2+}]\textsubscript{ER} steady-state levels in Bcl-2/Bax\((\alpha5-\alpha6)\)-transfected (73%) and Bcl-2-transfected cells (80%, Fig. 4B). In agreement with this result, in Bcl-2/Bax\((\alpha5-\alpha6)\)- and Bcl-2-transfected cells a comparable reduction was observed in the amplitude of the agonist-dependent [Ca\textsuperscript{2+}]\textsubscript{m} rises of mitochondria (79 and 73% of controls for Bcl-2 and Bcl-2/Bax \((\alpha5-\alpha6)\), respectively (Fig. 4C) and cytosol (85 and 91% of controls for Bcl-2 and Bcl-2/Bax\((\alpha5-\alpha6)\) (Fig. 4D)). Conversely, similarly to Bax, overexpression of the Bax/Bcl-2\((\alpha5-\alpha6)\) chimera modestly perturbed ER Ca\textsuperscript{2+} homeostasis (Fig. 4B) and greatly reduced the agonist-evoked [Ca\textsuperscript{2+}]\textsubscript{m} peak (68 and 54% of controls in Bax and Bax/Bcl-2\((\alpha5-\alpha6)\) (Fig. 4C)). The agonist-dependent [Ca\textsuperscript{2+}]\textsubscript{c} peak is also slightly diminished (90 and 83% of controls in Bax and Bax/Bcl-2\((\alpha5-\alpha6)\) (Fig. 4D)).

Altogether, these data strongly suggest that the putative pore-forming domains of Bcl-2 and Bax are not the key determinants of the different effect of the two proteins on Ca\textsuperscript{2+} homeostasis, as the two chimeric proteins maintain the effect on Ca\textsuperscript{2+} signaling of the native polypeptide and do not acquire that of the introduced \(\alpha5-\alpha6\) helices.

**Relationship between Ca\textsuperscript{2+} Dynamics and Apoptotic Regulation by Bcl-2 and Bax Proteins and the Bax/Bcl-2 Chimeras—**

We reported previously that Bcl-2 prevents mitochondrial damage caused by Ca\textsuperscript{2+} mobilizing apoptotic stimuli, such as ceramide, and protects cells from apoptosis (6). Conversely, Bax was proposed to exert a direct effect on mitochondria to promote apoptosis, with different putative mechanisms: (i) disturbing the mitochondrial membrane barrier function and (ii) binding to the pro-apoptotic Bcl-2 family proteins via its BH3 domain, thus inhibiting their function. Mitochondrial Ca\textsuperscript{2+} overload has been shown to promote the opening of the permeability transition pore with ensuing swelling and release of cytochrome c and pro-apoptotic factors into the cytosol (25). We previously reported that Ca\textsuperscript{2+} mobilizing apoptotic agents such as C\textsubscript{2}-ceramide induce alteration of mitochondrial structure (6). We hypothesized that reduction of mitochondrial Ca\textsuperscript{2+} uptake in Bax and Bax/Bcl-2\((\alpha5-\alpha6)\) mutant overexpressing cells could be the end result of a major morphological alteration of mitochondria structure, which might reduce the sites of close contact between ER and mitochondria and/or the driving force for organelle Ca\textsuperscript{2+} uptake. Thus we investigated whether the expression of Bax, Bcl-2, and the Bax/Bcl-2 chimeras affects mitochondrial structure and/or influences the alterations induced by ceramide, an apoptotic mediator shown to act through mitochondria in a Ca\textsuperscript{2+}-sensitive manner.

For this purpose, HeLa cells were transfected with the mitochondrial fluorescent marker mGFP either alone (controls) or co-transfected with Bcl-2, Bax, or the appropriate Bcl-2/Bax chimera. Then, organelle structure was evaluated at 36 h post-transfection using a high resolution digital imaging system. Acquired images were computationally deblurred as described under "Experimental Procedures." A, control cells; B, Bcl-2- and Bcl-2/Bax\((\alpha5-\alpha6)\)-expressing cells; C, Bax- and Bax/Bcl-2\((\alpha5-\alpha6)\)-expressing cells.

We then investigated the effects of Bax and the Bax/Bcl-2\((\alpha5-\alpha6)\) chimera. Fig. 5C shows representative images of the mitochondrial network visualized by mGFP in Bax- and Bax/Bcl-2\((\alpha5-\alpha6)\)-transfected cells. In comparison with control cells (Fig. 5A), a major mitochondrial morphological alteration (i.e. fragmentation of the network and swelling) was evident in Bax (43% of analyzed cells) and in Bax/Bcl-2\((\alpha5-\alpha6)\) (35% of analyzed cells) overexpressing cells (Fig. 5A). The remaining cells were characterized by a partial fragmentation of mitochondria.

The we evaluated the effect of the various chimeras on apoptosis. Firstly, we investigated whether the Bcl-2/Bax\((\alpha5-\alpha6)\) mutant also shared with Bcl-2 the capacity of protecting cells against the apoptotic effect of C\textsubscript{2}-ceramide. As reported previously (6), mGFP was co-transfected with the Bcl-2-related protein of interest, and after C\textsubscript{2}-ceramide treatment, the percentage of fluorescent cells was counted. If the protein has no (positive or negative) effect on apoptosis, transfected and non-transfected cells are equally sensitive to the apoptotic agent, and thus albeit the total number of viable cells is reduced, the percentage of transfected cells is the same as in controls. Conversely, the percentage of transfected cells increases if transfected cells are protected (as is the case of anti-apoptotic proteins) and decreases if the protein exerts the opposite effect. As reported previously (6), Bcl-2-transfected cells displayed an 15, and 31% in control, Bcl-2, and Bcl-2/Bax\((\alpha5-\alpha6)\) cells, respectively.

*Fig. 5. Effect of the expression of Bcl-2, Bax, and the Bcl-2/Bax chimeras on mitochondrial morphology. Analysis of the mitochondrial network structure was performed in HeLa cells transfected with mtGFP (Control) or co-transfected with mtGFP and Bax, Bcl-2, or Bcl-2/Bax chimera expression plasmids (see "Experimental Procedures"). The mitochondrial structure was evaluated 36 h post-transfection by visualizing mtGFP with a high resolution digital imaging system. Acquired images were computationally deblurred as described under "Experimental Procedures." A, control cells; B, Bcl-2- and Bcl-2/Bax\((\alpha5-\alpha6)\)-expressing cells; C, Bax- and Bax/Bcl-2\((\alpha5-\alpha6)\)-expressing cells.*
Finally, we evaluated the capacity of Bax and the Bax/Bcl-2(a5-a6) chimera to induce spontaneous apoptosis (Fig. 6B). In agreement with their similar effects on Ca\(^{2+}\) signaling (see Fig. 4) and on mitochondrial structure (see Fig. 5B), wild-type Bax and the Bax chimera with the Bcl-2 a5-a6 helices show a very similar capacity to induce apoptosis. Indeed, upon Bax and Bax/Bcl-2(a5-a6) expression, 25 \(\pm\) 4% and 19 \(\pm\) 5% of cells, respectively, showed morphological signs of spontaneous apoptosis, without the exogenous addition of apoptotic stimuli (Fig. 6B).

DISCUSSION

The link between dysregulation of calcium homeostasis and the control of apoptosis has been strengthened by a series of experimental observations converging into a coherent scheme. The most thoroughly investigated case is that of Bcl-2. The first evidence was obtained in cell clones stably expressing the oncprotein, and it indicated that Bcl-2 maintains ER Ca\(^{2+}\) filling and prevents the unloading of ER Ca\(^{2+}\) content caused by various pro-apoptotic conditions (e.g. treatment with the SERCA pump inhibitor, thapsigargin (26)). However, more recently, experiments with different probes and cell models demonstrated a reduction of ER Ca\(^{2+}\) level upon Bcl-2 expression (5, 27) and, conversely, a reduction when Bcl-2 endogenous expression was silenced (11). The partial emptying of agonist-sensitive Ca\(^{2+}\) stores and the ensuing reduction of cellular Ca\(^{2+}\) responses have been shown to be part of the protecting mechanism against apoptotic stimuli such as ceramide (6) and hydrogen peroxide or arachidonic acid (11). The investigation of pro- and anti-apoptotic proteins of viral origin then showed that the manipulation of Ca\(^{2+}\) signaling is not restricted to this important oncogene but may represent a more general mechanism in the control of apoptosis. Indeed, the anti-apoptotic Cox-sackie viral protein 2B has been shown to reduce ER Ca\(^{2+}\) levels (28). Interestingly, the pro-apoptotic protein X of hepatitis B (HBX) has been shown to enhance cellular responses to agonists not by altering ER Ca\(^{2+}\) levels but by inducing the caspase-dependent cleavage of the plasma membrane Ca\(^{2+}\) pump (29). In this scenario, it is expected that the pro-apoptotic members of the Bcl-2 family do not share with Bcl-2 itself the capacity of reducing ER Ca\(^{2+}\) levels or may even have the opposite function. Evidence that the latter is most likely the case was provided by Scorrano et al. (11) who have shown that gene ablation of Bax and Bak causes a reduction of ER Ca\(^{2+}\) levels and ensuing resistance to Ca\(^{2+}\)-dependent apoptotic stimuli, similar to what has been reported for Bcl-2 overexpression.

The effect of Bax on intracellular Ca\(^{2+}\) homeostasis was directly addressed in this paper, investigating (i) whether recombinant expression of Bax affected the ER Ca\(^{2+}\) levels and agonist-dependent responses of the cells and (ii) the structural basis for the different actions of Bcl-2 and Bax. Two expression systems were employed: (i) transient expression under the control of a constitutive promoter and (ii) a stable clone in which conditional expression of Bax was driven by a tetracycline-regulated promoter, and thus levels and timing of the expression could be more finely tuned. The combination of the two series of data provided significant information regarding the time course and dose dependence of the effect of Bax overproduction. The primary effect of Bax on Ca\(^{2+}\) homeostasis was evident in the conditional expression system, relatively soon after the induction of low level Bax expression. At this phase, still no significant increase in the number of apoptotic cells was detected, and thus the detected changes in Ca\(^{2+}\) homeostasis were not due to late secondary apoptotic alterations. Under these conditions, we observed an increase in ER Ca\(^{2+}\) levels
that correlated with an increase in mitochondrial Ca\(^{2+}\) loading upon challenging of cells with stimuli, causing the release of Ca\(^{2+}\) from the ER Cu\(^{2+}\) pool. These results agree with findings from Bax/Bak knock-outs (11). Furthermore, this observation is in agreement with the work by Snyder and co-workers (30) who demonstrated that the initial release of cytochrome c potentiates IP\(_3\)-mediated Ca\(^{2+}\) release, forming a positive feedback loop to activate the mitochondrial phase of apoptosis. Conversely, when Bax was transiently expressed attaining high protein level, as well as when Bax expression was prolonged in the inducible clone, no difference became detectable between the steady-state Ca\(^{2+}\) levels of controls and Bax-expressing cells. Under those conditions, two features dominated the general picture of Ca\(^{2+}\) signaling. The first was a drastic perturbation of mitochondrial three-dimensional structure and of key functional parameters, including mitochondrial membrane potential and the Ca\(^{2+}\) uptake capacity of the organelle. This effect also provides a possible explanation for the gradual decrease in ER Ca\(^{2+}\) loading; reduced ATP provision to the SERCA pumps could gradually impair Ca\(^{2+}\) (re)accumulation into the ER, thus bringing the [Ca\(^{2+}\)]\(_{\text{ER}}\) steady-state levels to those of a control cell. An alternative possibility can be presumed from the recent demonstration that the IP\(_3\) receptor includes a consensus site for caspase-3 cleavage, and the cleaved form of the receptor exhibits increased leakiness and reduced sensitivity to IP\(_3\) (31, 32). The latter finding would account not only for the reduction of steady-state [Ca\(^{2+}\)]\(_{\text{ER}}\), but also for the second obvious feature of the late apoptotic phenotype, i.e. the reduction of the Ca\(^{2+}\) release rate from the ER upon cell stimulation. In turn a smaller Ca\(^{2+}\) release results in the reduction of the amplitude of cytosolic Ca\(^{2+}\) responses (an effect that cooperates with the direct effect on mitochondria in causing the dramatic 30–50% reduction of mitochondrial [Ca\(^{2+}\)] peaks).

We then investigated whether the different effect on ER Ca\(^{2+}\) homeostasis of Bcl-2 and Bax depended on the properties of the putative pore-forming region. For this purpose, we employed two chimeras generated by us, in which the α-helices that were proposed to form the ion channel of Bcl-2(α5-α6) were mutually swapped between the two proteins (19). The prediction was that if the putative pore-forming region is the determinant of the effect on Ca\(^{2+}\) signaling, the α5-α6 helices would transfer this modulatory effect from the protein from which it is derived to the protein in which it is inserted. Thus, in transient expression experiments, one would predict that a Bcl-2 protein with the α5-α6 helices of Bax should induce no alteration of ER Ca\(^{2+}\) levels (as observed with Bax itself), whereas a Bax protein with the α5-α6 helices of Bcl-2 should partially empty the ER store. This was clearly not the case; the insertion of the α5-α6 helices of the other protein did not modify the primary effect on Ca\(^{2+}\) signaling of Bax and Bcl-2, implying that this effect does not depend on their putative pore-forming domain. Similarly, the two chimeras retained the effects on apoptosis of the original protein: the Bax protein with Bcl-2(α5-α6) helices is pro-apoptotic and causes perturbation of mitochondrial structure and function (including the drastic reduction of [Ca\(^{2+}\)]\(_{\text{mito}}\) responses), whereas the Bcl-2 chimera with Bax(α5-α6) protects against stimuli, such as ceramide, acting through a calcium and mitochondria-dependent pathway. Thus, one should conclude that (i) either the alteration of Ca\(^{2+}\) signaling is not due to the channel activity of these regions but depends on the modulation of the activity of other resident channels, or (ii) if an intrinsic channel activity is responsible for the effect, a different regulation occurs in the two proteins. In all cases, the key determinant of the signaling specificity rests in a part of the molecules that is distinct from their putative pore-forming domain (the α5-α6 helices). Similar conclusions were drawn from two recent works regarding the structure/function relationship of Bax and Bcl-2. Namely, Bax(α5-α6) was shown to be essential for Bax-mediated cytochrome c release (33), but the modulation of the adenine nucleotide translocase, taking part in the mitochondrial permeability transition pore, presumably requires interaction of other parts of the Bax and Bcl-2 proteins (34). Moreover, in linking Bcl-2 to cellular Ca\(^{2+}\) signaling through a mechanism independent of the pore-forming domain, our results are sup-
ported by the finding of Bassik et al. (35), who showed that the ability of Bcl-2 to lower [Ca\(^{2+}\)]\(_{\text{ER}}\) and to protect against Ca\(^{2+}\)–dependent death stimuli depends on a regulatory event, i.e. on the stimulation of IP\(_3\) receptor phosphorylation, leading to the consequent increase of the leakiness of the ER resident Ca\(^{2+}\) channel.

The general picture emerging from these results (Fig. 7) is that Bax and Bcl-2 both have an effect on Ca\(^{2+}\) signaling that does not depend on the region proposed to form an ion channel but resides in a yet unidentified segment of the proteins. Moreover, it is unclear from these studies whether the ability of Bcl-2 and Bax to regulate ER Ca\(^{2+}\) is an intrinsic property of these proteins versus a manifestation of a regulatory effect on other proteins. As reported previously, Bcl-2 reduces ER Ca\(^{2+}\) levels, and consequently it moderates the efficacy of apoptotic mediators that use Ca\(^{2+}\) signals (and the involvement of mitochondria as downstream effectors) as a potentiation-commitment factor. Conversely, Bax enhances the loading of the ER Ca\(^{2+}\) store and thus boosts the Ca\(^{2+}\) load to which the apoptotic effector systems (including mitochondria) are exposed upon physiological and/or pathological challenges. This effect of Bax coincides with gross perturbation of mitochondrial structure and function and finally, later in apoptotic progression, to the development of an altered signaling phenotype, which includes impaired ER Ca\(^{2+}\) release upon cell stimulation and thus reduction of cellular Ca\(^{2+}\) signals. Although much remains to be understood about the molecular targets of these signaling alterations, these results strongly reaffirm the notion of the involvement of Ca\(^{2+}\) in the control of apoptosis and suggest possible opportunities for developing pharmacological modulators of this pathophysiological event.

REFERENCES